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Comparative study between the polysaccharide-based Chiralcel OJ and Chiralcel OD CSPs in chromatographic enantioseparation of imidazole analogues of Fluoxetine and Miconazole

The enantiomeric separation of a series of imidazole analogues of Fluoxetine and Miconazole endowed with potent antifungal activity was performed using cellulose tris(4-methylbenzoate) (Chiralcel OJ) and cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD) as chiral stationary phases. Binary mixtures of *n*-hexane and alcohol as well as pure alcohols (ethanol or 2-propanol) were used as eluents. The enantiomer elution order was monitored by chiroptical detectors based on on-line optical rotation and circular dichroism measurements. For some of the compounds studied very high enantioseparation factor values ($\alpha > 7$) on Chiralcel OJ CSP were observed. In order to study the chiroptical characteristics of the two most biologically active compounds, chromatographic resolutions were carried out on a semipreparative scale. Assignment of the absolute configuration was empirically established by comparing the CD spectra of the separated enantiomers with those obtained from the enantiomers of Miconazole.

Key Words: Cellulose chiral stationary phases; Circular dichroism detection; Optical rotation; Absolute configuration; Antifungal agents

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1 Introduction

It is widely known that individual enantiomers of chiral drugs can have very different pharmacological and toxicological activities. This has led to an increased demand for chiral pharmaceuticals as pure enantiomers and more stringent rules for chiral analysis by regulatory agencies [1].

In the last two decades, the chiral HPLC method has attained increasing prominence in the direct analytical and preparative separation of enantiomers. The reasons for this popularity are to be found in the inherent advantages of the chromatographic method (rapid analysis, reproducibility, flexibility) and in the growing development and commercialisation of new chiral stationary phases. Nowadays, approximately 170 chiral stationary phases are commercially available [2]. The wide spectrum of suitable chiral selectors include very different molecular species such as amino acid derivatives [3–5], crown ethers [6], cyclodextrins [7], glycopeptides [8], proteins [9], and synthetic [10] and modified natural polymers [11].

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Abbreviations: CSP, chiral stationary phase; DEA, diethylamine; CD, circular dichroism; OR, optical rotation; UV, ultraviolet.

Polysaccharide-based CSPs coated onto a silica support, originally introduced by Shibata et al. [12] and Okamoto [13–15], are semisynthetic polymers substantially obtained by conversion of alcoholic groups of monosaccharide units into arylcarboxylate or arylcarbamate moieties. Among the numerous derivatives of natural polysaccharides synthesised, cellulose tris(4-methylbenzoate) (Chiralcel OJ) and cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD) represent two of the most effective chiral selectors for HPLC because of their excellent resolving ability for a wide variety of chiral compounds of pharmaceutical and synthetic interest [16–19].

In the present study, we report on the enantiodiscriminating capability of OJ and OD CSPs towards a set of novel racemic compounds **3–10** endowed with potent antifungal activity, imidazole analogues of the antidepressant agent Fluoxetine [20] and the antifungal agent Miconazole (**Figure 1**, compounds **1**and **2**) [21–23]. In this context, the influence of structural features of the selectands and of the selectors on enantioselectivity is discussed. The chiral resolving ability of both cellulose-based CSPs was apparent in the presence of typical normal-phase *n*-hexane-alcohol mixtures or pure alcohols (ethanol or 2-propanol) used as eluents. The enantiomer elution order was monitored by chiroptical detectors based on on-line optical rotation (OR) or circular dichroism (CD) measurements.

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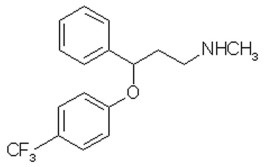
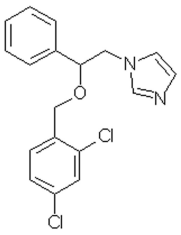
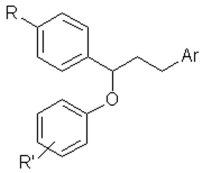


1- Fluoxetine



2- Miconazole



3-10

Compound	R	R'	Ar
3	H	4-NO_2	
4	H	2,4-Cl	
5	H	2,4-F	
6	H	4-CF_3	
7	H	4-CH_3	
8	H	2,4-Cl	
9	H	4-Cl	
10	Cl	2,4-Cl	

Figure 1. Structures of the chiral analytes **1–10**.

Two of the most biologically active compounds (compounds **4** and **7**) and compound **2** (Figure 1) were resolved on a semipreparative scale by using Chiralcel OD and Chiralcel OJ as CSPs. The chiroptical properties of the isolated enantiomers were determined. The absolute configuration of stereoisomers of the analyte **4** was empirically assigned by comparing their CD spectra with those of stereoisomers of the structural analogue Miconazole.

2 Experimental

2.1 Chemicals

Compounds **3–10** have been previously synthesised [20]. All compounds were dissolved in ethanol in a concentration of about 0.4 mg/mL. Ethanol and *n*-hexane were purchased from Merck (Darmstadt, Germany), 2-propanol was obtained from Panreac (Barcelona, Spain). DEA was obtained from Aldrich (Gillingham, UK). All reagents were HPLC grade. (−)-(*R*)- and (+)-(*S*)-Fluoxetine hydrochloride, and the hold-up time marker 1,3,5-tri-*tert*-butylbenzene were purchased from Sigma (St. Louis, MO, USA); Miconazole base was kindly supplied by Janssen-Cilag S.p.A. (Milano, Italy).

2.2 Chromatographic conditions

About 20 μL of each of the solutions was injected onto a HPLC system consisting of a LabService Analytica solvent delivery pump (Model LabFlow 3000), a Rheodyne injector (Cotati, CA) with a 20 μL loop, a Jasco CD 2095 Plus Model chiroptical detector (Japan Spectroscopy, Tokyo, Japan). The columns used were Chiralcel OJ (cellulose tris-4-methylbenzoate, 10 μm, 250 × 4.6 mm and 250 × 10 mm ID) and Chiralcel OD (cellulose tris-3,5-dimethylphenylcarbamate, 10 μm, 250 × 4.6 mm ID and 250 × 10 mm ID); they were obtained from Daicel Chemical Industries, Tokyo, Japan. The mobile phases used in this study were: *n*-hexane/2-propanol/DEA and *n*-hexane/ethanol/DEA (50 : 50 : 0.1 and 70 : 30 : 0.1, *v/v/v*), 2-propanol/DEA and ethanol/DEA (100 : 0.1, *v/v*). The mobile phases were filtered through a Millipore membrane filter (0.45 μm) Millipore (Yonezawa, Japan) and degassed before use. The flow rate of the mobile phase was 1 mL/min with the normal phase eluents, 0.3 and 0.5 mL/min in the polar organic conditions. Chromatographic signals were collected using UV and CD detectors both operating at the analytical wavelength of 280 nm.

Semipreparative enantioseparations of **2**, **4**, and **7** were performed by using a Perkin Elmer pump (Model 410, Perkin-Elmer, Norwalk, CT, USA), a Waters Model 484 tuneable absorbance detector, operating at 300 and 310 nm, a Rheodyne injector (Cotati, CA) with a 2 mL loop. The signal was acquired and processed by Millennium 2010 software. The mobile phase used was ethanol/DEA (100 : 0.1, *v/v*). The flow rate was 2 mL/min. The semipreparative column was loaded with 1000 μL of solutions of 10 mg/mL of each compound. After each semipreparative chromatographic run, fractions corresponding to the single enantiomers were pooled and evaporated. The collected fractions of the single enantiomers were analysed on a chiral analytical OJ or OD column to determine their enantiomeric

excess (e.e.). The enantiomer elution order of **4** and **7** on both OD and OJ CSPs was monitored by a Perkin Elmer Model 241 polarimeter (Norwalk, CT, USA) equipped with Hg/Na lamps and a 40 μL flow cell.

All the experiments were carried out at 25°C. A Perkin-Elmer 101 LC Oven (Norwalk, CT, USA) controlled the column temperature.

2.3 Equipment

Specific rotations of the single enantiomers obtained by semipreparative chromatography of compounds **2**, **4**, and **7**, and dissolved in ethanol, were measured on a Perkin-Elmer 241 polarimeter equipped with a Na lamp operating at 589 nm. The volume of the measuring cell was 1 mL and the length of the optical path 10 cm. The system was thermostated at 23°C.

CD spectra were recorded on a Jasco J-710 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) using a 1 cm quartz cell. CD measurements were performed by using samples dissolved in ethanol at a concentration of about 0.1 mg/mL. The system was thermostated at 25°C.

3 Results and discussion

3.1 Chromatographic enantioseparation of compounds 1–10

Figure 1 shows the structures of the novel azole antifungal agents **3–10**. They were obtained by replacing the methylamino moiety of the aminopropane chain of Fluoxetine (compound **1**) with an imidazole (compounds **3–7**, **9**, and **10**) or a triazole (compound **8**) ring. Furthermore, different substituents were introduced in the phenyl and phenoxy groups of the reference Fluoxetine. Enantiomeric separations of **1–10** were performed on two cellulose-based Chiralcel OD and Chiralcel OJ CSPs by using normal-phase *n*-hexane-alcohol mixtures or pure alcohols, containing 0.1% of DEA, as eluents. **Table 1** and **Table 2** summarise the chromatographic results obtained.

Inspection of Table 1 reveals the high chiral recognition ability of the OJ CSP towards the analytes investigated, in both normal phase and polar organic modes. Thus, among 10 chiral analytes only Fluoexetine was not resolved at all and good enantioseparations could be observed for the other ones ($\alpha > 1.15$), under at least one of set of conditions employed.

Ethanol to which DEA had been added was, in general, the most suitable mobile phase. Thus, for example, compound **4** was not resolved when 2-propanol was used in admixture with *n*-hexane and DEA, while an enantioselectivity factor value of 1.50 was observed in the presence of the *n*-hexane-ethanol-DEA mixture. Exceptions were represented by compounds **5** and **8** which were better resolved in *n*-hexane-2-propanol ($\alpha = 1.29$ and 1.64) and in pure 2-propanol eluent ($\alpha = 1.23$ and 1.40) than in *n*-hexane-ethanol ($\alpha = 1$ and 1.43) or in pure ethanol ($\alpha = 1.19$ and 1.31). Three out of the ten analytes (compounds **6**, **7**, and **9**) were separated with large enantiose-

Table 1. Capacity factors (k), selectivity factors (α),and resolution factors (R_s) for the compounds investigated **1–10** obtained on Chiralcel OJ (250 × 4.6 mm ID) using *n*-hexane/2-propanol/DEA (A) and *n*-hexane/ethanol/DEA (B) (50:50:0.1, *v/v/v*), 2-propanol/DEA (C) and ethanol/DEA (D) (100:0.1, *v/v*) as mobile phases with 1 mL/min (A and B), 0.3 mL/min (C) and 0.5 mL/min (D) flow rates. UV and CD detectors at 280 nm; temperature 25°C.

Compound	Eluent A			Eluent B			Eluent C			Eluent D		
	k_1[a]	α[b]	R_s[c]	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
1	1.84[d]	1	nr	1.70[e]	1.00	nr	0.16	1.00	nr	0.07	1.00	nr
2	1.81 (+)[f]	1.28	1.62	0.93 (+)	1.93	5.06	2.22 (+)	1.23	1.22	1.06 (+)	1.80	4.74
3	5.02 (+)	1.65	2.08	2.03 (+)	2.49	6.17	1.53 (+)	1.94	3.27	0.86 (+)	2.64	4.76
4	2.86	1	nr	1.06 (−)	1.50	2.96	1.16 (−)	1.21	0.87	0.77 (−)	1.60	2.25
5	4.44 (−)	1.29	1.19	2.30	1.00	nr	0.44 (−)	1.23	0.88	1.24 (+)	1.19	1.24
6	5.38 (−)	8.46	7.86	2.24 (−)	8.71	21.30	3.30 (−)	7.95	8.57	1.46 (−)	9.86	32.76
7	5.24 (−)	9.81	6.85	2.22 (−)	8.79	20.92	3.28 (−)	8.12	8.89	1.46 (−)	10.06	26.69
8	2.23 (+)	1.64	2.98	0.97 (+)	1.43	2.30	1.48 (+)	1.40	1.08	0.69 (+)	1.31	1.20
9	3.29 (−)	5.32	5.68	1.10 (−)	6.31	18.87	2.34 (−)	4.42	4.33	0.67 (−)	7.24	19.02
10	1.90	1	nr	0.66 (−)	1.24	1.08	0.83	1.00	nr	0.51 (−)	1.15	0.74

[a] Retention factor for the first eluting enantiomer.
[b] Enantioselectivity factor.
[c] Resolution factor.
[d] Eluent: *n*-hexane/2-propanol/DEA (98.5:1.5:0.1, *v/v/v*); flow rate: 1 mL/min.
[e] Eluent: *n*-hexane/ethanol/DEA (98.5:1.5:0.1, *v/v/v*); flow rate: 1 mL/min.
[f] Sign of circular dichroism-based detector at 280 nm.

Table 2. Capacity factors (k), selectivity factors (α) and resolution factors (R_s) for the compounds investigated **1**–**10** obtained on Chiralcel OD (250 × 4.6 mm ID) using *n*-hexane/2-propanol/DEA (A) and *n*-hexane/ethanol/DEA (B) (50:50:0.1, *v/v/v*), 2-propanol/DEA (C) and ethanol/DEA (D) (100:0.1, *v/v*) as mobile phases with 1 mL/min (A and B), 0.3 mL/min (C), and 0.5 mL/min (D) flow rates. UV and CD detectors at 280 nm; temperature 25°C.

Compound	Eluent A			Eluent B			Eluent C			Eluent D		
	k_1[a)]	α[b)]	R_s[c)]	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
1	4.00[d]	1.00	nr	1.94(+)[e, f)]	1.06	0.70	0.32	1.00	nr	0.27	1.00	nr
2	1.08	1.00	nr	0.81 (−)	1.22	1.45	1.60	1.00	nr	0.60	1.00	nr
3	7.21 (−)	1.82	4.37	2.79 (−)	1.59	4.13	1.81 (−)	1.46	2.92	0.63 (−)	1.56	2.40
4	4.11 (+)	2.41	7.19	1.69 (+)	2.52	3.98	8.52 (+)	1.53	4.96	0.68 (+)	2.10	4.57
5	4.04 (−)	1.83	5.24	1.56 (−)	1.81	5.11	7.12 (−)	1.33	3.24	0.14 (−)	3.85	8.84
6	4.41 (+)	1.34	2.81	1.73 (+)	1.36	2.48	1.62 (+)	1.33	2.58	0.66 (+)	1.45	2.20
7	4.42 (+)	1.39	2.51	1.75 (+)	1.40	1.85	1.62 (+)	1.34	2.58	0.65 (+)	1.45	2.15
8	3.86 (+)	1.72	4.43	1.81 (+)	2.12	3.54	1.70 (+)	1.53	3.01	0.65 (+)	1.86	3.21
9	3.51 (+)	1.44	3.33	1.68 (+)	1.49	3.03	1.38 (+)	1.30	2.12	0.55 (+)	1.47	1.94
10	3.56 (−)	1.20	1.24	1.35 (−)	1.29	1.79	1.10 (−)	2.04	5.20	0.44 (+)	2.19	5.67

a) Retention factor for the first eluting enantiomer.
b) Enantioselectivity factor.
c) Resolution factor.
d) Eluent: *n*-hexane/2-propanol/DEA (98.5:1.5:0.1, *v/v/v*); flow rate: 1 mL/min.
e) Eluent: *n*-hexane/ethanol/DEA (98.5:1.5:0.1, *v/v/v*); flow rate: 1 mL/min.
f) Sign of circular dichroism-based detector at 280 nm.

paration factors in all conditions used, especially in pure ethanol eluent ($\alpha > 7$).

The enantioseparation abilities of Chiralcel OD CSP appeared to be in many cases complementary with respect to the analogous cellulose-based OJ CSP. Thus, for instance, compound **4** was not separated on the Chiralcel OJ CSP with a mixture of *n*-hexane-2-propanol as eluent, while good enantioselectivity factors were obtained in all the tested conditions when a Chiralcel OD column was used as stationary phase (see **Table 2**). On the contrary, compound **2** was well separated on Chiralcel OJ CSP under all the conditions tested, with enantioselectivity factors ranging from 1.23 to 1.93, but only with a mixture of *n*-hexane-ethanol (α = 1.22) on Chiralcel OD CSP. Fluoxetine (compound **1**) was not enantioselectively recognised under polar organic conditions and in mixture *n*-hexane-2-propanol, while a slight enantioseparation was observed in presence of *n*-hexane-ethanol-DEA eluent. Compound **5**, not resolved on the Chiralcel OJ column in combination with a mixture of *n*-hexane-ethanol, was resolved with a good enantioseparation factor (α = 1.81) on the Chiralcel OD column. A higher enantioselectivity factor was obtained for the same compound when ethanol was used as eluent (α = 3.85). No enantiomeric separation of compound **10** was obtained with a combination CSP-eluent OJ-2-propanol, while total resolution was observed on replacing the ester-type OJ CSP with the carbamate-type OD CSP.

On following the comparative study between the two cellulose-based CSPs employed, it emerges that although Chiralcel OD allowed the resolution of all analytes, in some circumstances lower enantioselectivity factors were achieved than on the OJ CSP. Thus, for compounds **6**, **7**, and **9** α values ranged from 4.42 to 10.06 on Chiralcel OJ CSP (Table 1) and from 1.30 to 1.49 on Chiralcel OD CSP (Table 2). Representative chromatograms of compound **6** on both Chiralcel OJ and Chiralcel OD CSPs are shown in **Figure 2**.

The distinctive chiral recognition ability of cellulose based CSPs depends on the structural features of polymeric selectors. X-ray and NMR analysis [24–25] and computational simulations [14, 26] have clarified that the tris(phenylcarbamate) of cellulose has a left-handed 3/2 helical conformation and the glucose moieties are regularly arranged along the helical axis. The chiral recognition ability of cellulose derivatives has been attributed to the degree of insertion of the enantiomers in the chiral cavities of the CSP.

The most important adsorbing sites for chiral recognition on arylcarbamate and alkylbenzoate derivatives of cellulose are probably the polar carbamate and ester moieties, respectively. These residues are both capable of interacting with enantiomers through hydrogen bonding: the carbamate-derivative OD with donor hydrogen bonding NH groups and acceptor hydrogen bonding C═O groups,

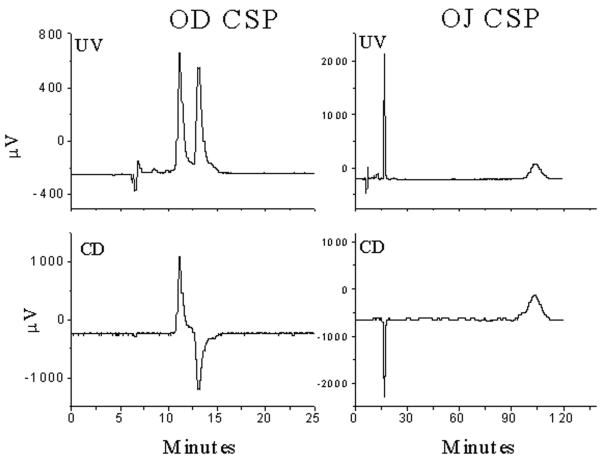


Figure 2. Chromatograms of compound **6** with UV (280 nm) (top) and CD (280 nm) (bottom) detection. Left: Column: Chiralcel OD (250 × 4.6 mm ID); eluent: ethanol/DEA (100 : 0.1, *v/v*); flow rate: 0.5 mL/min; column temperature: 25°C. Right: Column: Chiralcel OJ (250 × 4.6 mm ID); eluent: ethanol/DEA (100 : 0.1, *v/v*); flow rate: 0.5 mL/min; column temperature: 25°C.

while the corresponding benzoate-derived OJ only with acceptor hydrogen bonding C═O groups.

The enantioseparation process involves the sum of multivariate and synchronous interactions between selectand and active sites of polymeric chiral stationary. Besides hydrogen bonding, dipole-dipole interactions, π-π interactions, and steric repulsion are strictly involved in the stability of transient selectand-selector complexes [13, 16].

The intermolecular forces involved in the chiral recognition mechanism depend not only on the nature of the stationary phase but also on the nature of analyte. Compounds **2–10**, not being capable of forming hydrogen bond, interact with the multiple sites of OJ CSP through alternative interactions.

This distinction seems to induce a different chiral recognition ability between OD and OJ CSPs in normal phase and polar organic conditions.

Alteration of number, nature, and position of substituents in the phenyl ring and phenoxy group leads to differences in electronic density and accessibility to analytes of the chiral grooves of the CSPs.

On replacing the electron-withdrawing group CF_3 (compound **6**) by an electron-pushing group CH_3 (compound **7**), no significant alteration in chiral recognition ability of OJ CSP was observed. In contrast, the substitution of the CF_3 moiety by a NO_2 group (compound **3**) or the insertion of a second substituent on the same aryloxy group (compounds **4** and **5**) produced a consistent lowering in enantioselectivity.

Compound **10** differs from compound **4** in the insertion of a chlorine atom on the phenyl ring. On the Chiralcel OJ column, the enantiomers of compound **4** were separated with enantioselectivity factors of 1.21 and 1.60, in the presence of 2-propanol and ethanol with added DEA, respectively. On using the same CSP-eluent combinations, compound **10** was not resolved in the presence of 2-propanol and a lower enantioselectivity ($\alpha = 1.15$) was obtained in presence of ethanol.

The OD CSP was more effective in the resolution of compound **10** with respect to **4** when polar organic eluents were used ($\alpha = 2.04$ and 2.19 for compound **10** versus 1.53 and 2.10 for compound **4**), but less effective when used with normal phase eluents ($\alpha = 1.20$ and 1.29 for compound **10** and 2.41 and 2.52 for compound **4**).

This probably means that the enantioseparation factors of **1–10** obtained on both cellulose-based CSPs are not influenced by π-π interactions between aryl or aryloxy moieties of solute and 3,5-dimethyl group of selector, but other forces may play a more important role in the formation of the analyte-CSP complex.

It appears interesting to note that a consistent increase in retention on both cellulose-based CSPs was achieved by replacing the methylamino moiety of the aminopropane chain of Fluoxetine with an azole group (Table 1 and Table 2). The longer interaction of the azole derivatives relative to Fluoxetine was followed by an improvement in chiral recognition. Thus, the enantiomers of Fluoxetine were poorly stereoselectively recognised by OD CSP, while both cellulose-based CSPs demonstrated a high enantiomer resolving ability for compounds **2–10**. This finding led to identification of the electron-rich azole moiety as an important site for the enantiorecognition mechanism.

As shown in Table 1 and Table 2, the alcohol (ethanol or 2-propanol) used in normal phase and polar organic eluents had in the same cases (Table 1 and Table 2, compounds **4**, **5**, and **10**) a noticeable influence on the chiral selectivity of polysaccharide-based CSPs.

These data support the argument that different alcohols can induce a peculiar CSP structural variation and consequently can provide different chiral selectivity for an enantiomeric pair [27].

The chiral recognition ability of polysaccharide-based CSPs is generally thought of as the consequence of the formation of CSP-solute complexes, whose stability depends on the degree of inclusion of the enantiomers in the chiral cavities of the higher order structure of the CSP. The difference in properties between ethanol and 2-propanol (such as solvating capacity towards the CSP and analyte, degree of incorporation into the CSP) may lead to a different stereo environment of the chiral cavities in the CSP and significantly influence the inclusion process of enantiomers.

Table 3. Chromatographic, quantitative, and polarimetric analysis of the first fraction F1 and second fraction F2 recovered during the enantioseparation of Miconazole (**2**), and Fluoxetine derivatives **4** and **7**.

Compound	SR[a)]	Yield [%]	F1 e.e [%]	$[\alpha]_D^{23}$	Yield [%]	F2 e.e. [%]	$[\alpha]_D^{23}$
2	10	90	>99.0	+60 (*c* = 0.4, EtOH)	80	98.9	−59 (*c* = 0.4, EtOH)
4	20	70	95.0	+10 (*c* = 0.1, EtOH)	80	98.0	−12 (*c* = 0.1, EtOH)
7	10	90	>99.0	−32 (*c* = 0.4, EtOH)	85	>99.0	+37 (*c* = 0.4, EtOH)

Experimental conditions used for semipreparative enantioseparations: Columns: Chiralcel OJ (**2**) and Chiralcel OD (**4** and **7**) 250 × 10 mm ID; Mobile phase: ethanol + 0.1% DEA (D); Flow rate: 2 mL/min; Wavelength of UV detector: 310 nm (**2** and **4**) and 300 nm (**7**); Temperature: 25°C (**2** and **7**) and 35°C (**4**); SR[a)]: semipreparative run: mg.

3.2 Semipreparative enantioseparation of 2, 4, and 7

The preparative chromatographic resolution of racemic mixtures is becoming a standard approach to obtain individual enantiomers in high purity. Generally, the coated polysaccharide-based chiral stationary phases need hydrocarbon-alcohol mixtures or pure polar solvents as mobile phase. In this work we used polar organic conditions with the aim of increasing the solubility of racemates and shorting the analysis times, requirements which are very important especially for high throughput separations in pharmaceutical analysis [28–30].

Two of the most biologically active compounds (compounds **4** and **7**) [20] of the presented series of novel antimycotic agents and compound **2** were resolved on a semipreparative scale by using Chiralcel OD and Chiralcel OJ as CSPs.

Table 3 summarises the chromatographic operating conditions employed in the enantioseparation of **2**, **4**, and **7** on the mg-scale, and the chromatographic and polarimetric data pertinent to each enantiomer that was collected in a single fraction.

With a mixture of ethanol-DEA 0.1% as mobile phase, resolutions of 10 mg of racemic compounds **2** and **7** on OJ and OD CSPs, respectively, and 20 mg of **4**, on OD CSP, were achieved within 20 minutes. Enantiomeric excess (e.e.) values ranging from 94.73% to > 99.00% and yields 80% were obtained.

Polarimetric analysis indicated that the first eluted enantiomer of compound **4** on the OD CSP rotated polarised light in the positive direction in ethanol solution at 589 nm ($[\alpha]_D^{23}$ = +10). A reversal of sign of the specific rotation was observed for the first eluted enantiomer of compound **7** on the OD CSP, in the same experimental conditions ($[\alpha]_D^{23}$ = −32). The less and more retained enantiomers of Miconazole on OJ CSP were dextrorotatory and levorotatory, respectively (Table 3).

3.3 Circular dichroism spectra of enantiomers of 1, 2, 4, and 7

The stereochemical assignment may be empirically supported by comparing the off-line CD spectra of enantiomers of unknown configuration with those of a structurally related compound of known configuration. As reported in **Figure 3**, the CD spectrum of (+)-(*S*)-enantiomer of Miconazole [21] showed an intense positive Cotton effect at 230 nm, a broad and weak positive band at around 270 nm CD, and a negative band near 236 nm. For the corresponding (−)-(*R*)-**2** enantiomer the CD spectrum was inverted, as expected. The CD spectra of (−)-enantiomer of **4** exhibited a shape strictly correlated to that of the (+)-(*S*)-**2** stereoisomer in the spectral range of 225–250 nm, but a very broad and slightly negative band in the range of 250–300 nm. Based on a comparison with the reference CD spectra of enantiomers of **2** of known absolute configuration, the (*S*)-configuration was assigned to the (−)-**4** enantiomer.

A direct correlation CD/absolute configuration was not possible for the compound **7** due to the different chromophores contributing to the CD spectra with respect to the reference compound **2**. In the same way, Fluoxetine was also ineffective as reference for the empirical stereochemical assignment of absolute configuration of **7**.

3.4 Elution order

Enantiomer elution order is an important topic in analytical as well as in preparative scale chiral HPLC. Although it is difficult to control the enantiomer elution order on CSPs if no enantiopure reference compound is available, different ways of achieving this aim have proved to be effective [31–32]. Chiroptical detectors in combination with a conventional UV detector offer significant advantages in this particular study. In the present work, the elution order of the enantiomers of compounds **1–10** was established by using a CD detector operating on-line at 280 nm.

On going from OD to OJ CSP, all chiral analytes with the exception of **8** exhibited inversion of their enantiomer elu-

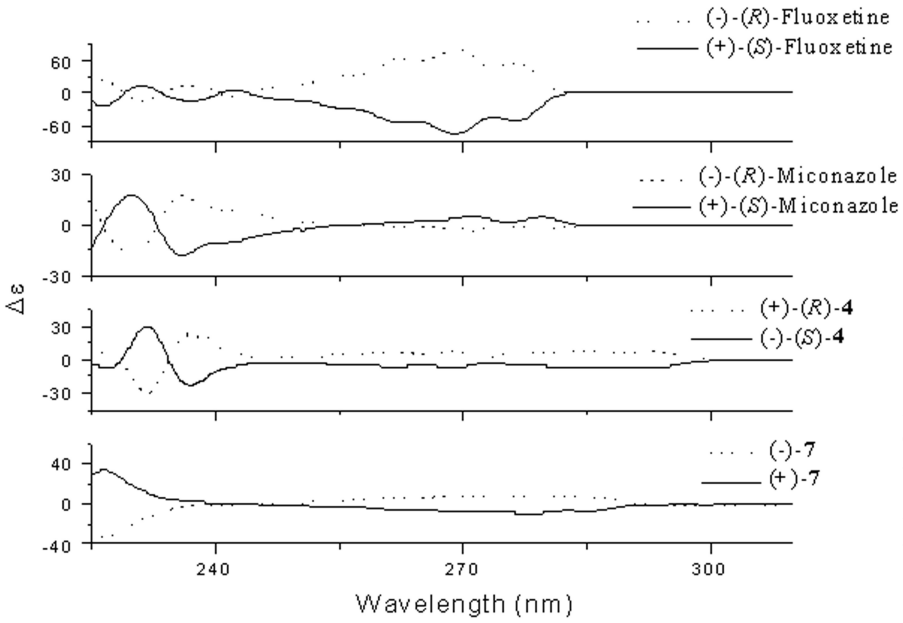


Figure 3. Circular dichroism (CD) spectra of the enantiomers of the compounds **1**, **2**, **4**, and **7** in ethanol at 25°C. Dashed and solid traces correspond respectively to the first and second eluted enantiomer on OD and OJ CSPs.

tion order. For example, as reported in Figure 2, the sign of CD at 280 nm of the first eluted enantiomer of compound **6** was negative on using the OJ/ethanol experimental combination, whereas it was positive with the OD/ethanol combination. These results suggest that a change in the enantiorecognition process, on transition from the carbamate-type OD CSP to the ester type OJ CSP, must take place for the aforementioned compound. The alternative active sites of selectors, ester or carbamate moieties, 4-methylphenyl or 3,5-dimethylphenyl groups, may interact in different ways with analytes and dramatically affect the enantiorecognition properties of the two polysaccharide-based CSP. In addition, the findings of this study confirm that, in some circumstances, the elution order of an enantiomeric pair may be reversed by changing the nature of the chiral stationary phase [33].

At the same time, on-line CD measurements are ineffective for comparing the elution order of enantiomers of different chiral analytes on the same CSP. In fact, the different on line-CD signs of two analytes can be due to a different contribution of functional groups to CD spectra or to a change of the elution order induced by an altered chiral recognition mechanism. On analysing the off-line CD spectra of compounds **2** and **4** in the 250–300 nm range (Figure 3), it was clear that the apparent inversion of elution order established by on-line CD-sign at 280 nm for the first eluted enantiomer (positive for (+)-(*S*)-**2** and negative for (−)-(*S*)-**4**, Table 1) was only due to an inversion of Cotton-effect. The same apparent inversion of elution order was also observed for Fluoxetine and Miconazole (compounds **1** and **2**) on OD CSP (Table 2).

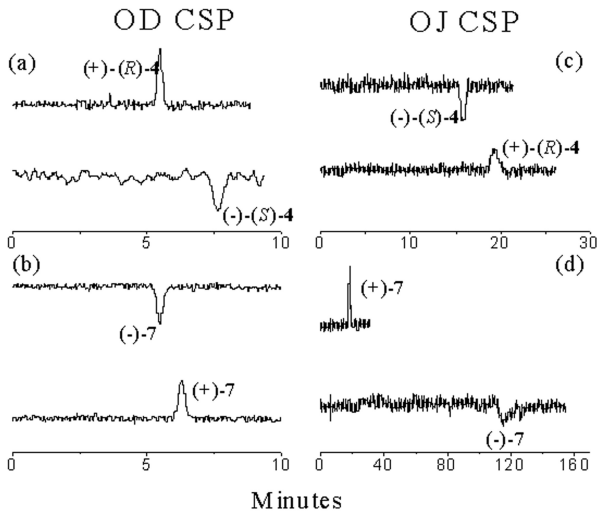


Figure 4. Polarimetric chromatograms of the enantiomers of **4** (top) and **7** (bottom). Top: Columns: Chiralcel OD (a) and Chiralcel OJ (c); eluent: ethanol; flow rate: 1 mL/min (a) and 0.4 mL/min (c); temperature: 25°C. Bottom: Column: Chiralcel OD (b) and Chiralcel OJ (d); eluent: ethanol; flow rate: 1 mL/min (b) and 0.4 mL/min (d); temperature: 25°C. Detection wavelength: 365 nm.

The elution order of compounds **4** and **7** isolated on a semipreparative scale was checked by employing the single enantiomers and a detector based on on-line optical rotation measurements. Because of low optical activity of the analytes investigated (Table 3), an amount of about 1 mg of each enantiomer in ethanol solution was injected during chiral HPLC. As reported in **Figure 4**, analytes **4** and **7** exhibited opposite enantiomer elution order on OD

and OJ CSPs using ethanol as eluent. The carbamate-type OD CSP showed preferential retention of the (−)-**4** and (+)-**7** enantiomer. The opposite enantiomer elution order was observed on OJ CSP. Thus, by replacing the chlorine atoms of the aryloxy portion of compound **4** by a methyl substituent (compound **7**), the specific rotation value of the second eluting enantiomer on OD CSP passed from −12 to +37 in ethanol (Table 3).

The reported example emphasizes how the deduction of the elution order solely based on the sign of CD and optical rotation, especially in cases of low optical activity, could lead to mistaken conclusions.

4 Concluding remarks

In this work the different enantioseparation capabilities of two cellulose-based CSPs towards new compounds endowed with antifungal activity has been demonstrated. The chiral HPLC method conducted on a semipreparative scale has been useful in characterizing the chiroptical properties of some compounds. The results achieved in this study can probably be used as a reference for other work.

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References

[1] FDA Policy, *Statements for the Development of New Stereoisomeric Drugs*. FDA, Rockville, MD, 1992.

[2] T.E. Beesley, R.P. Scott, *Chiral Chromatography*, Wiley-VCH, New York 1998.

[3] W.H. Pirkle, J.M. Finn, J.L. Schreiner, B.C. Hamper, *J. Am. Chem. Soc.* **1981**, *103*, 3964–3966.

[4] G. Gargaro, F. Gasparrini, D. Misiti, G. Palmieri, M. Pierini, C. Villani, *Chromatographia* **1987**, *24*, 505–509.

[5] I.A. Yamskov, B.B. Berezin, A. Davankov, Yu.A. Zolotarev, I.N. Dostavalov, N.F. Myasoedov, *J. Chromatogr.* **1981**, *217*, 539–543.

[6] G.D.Y. Sogah, D. Cram, *J. Am. Chem. Soc.* **1976**, *98*, 3038–3041.

[7] D.W. Armstrong, W. DeMond, *J. Chrom. Sci.* **1984**, *22*, 411–415.

[8] D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J.R. Chen, *Anal. Chem.* **1994**, *66*, 1473–1484.

[9] J. Hermansson, *J. Chromatogr.* **1983**, *26*, 71–77.

[10] Y. Okamoto, T. Nakano, *J. Am. Chem. Soc.* **1980**, *102*, 6356–6358.

[11] Y. Okamoto, R. Aburatani, K. Hatada, *Bull. Chem. Soc. Jpn.* **1990**, *63*, 955–957.

[12] T. Shibata, I. Okamoto, K. Ishii, *J. Liq. Chromatogr.* **1986**, *9*, 313–340.

[13] Y. Okamoto, Y. Kaida, *J. Chromatogr. A* **1994**, *666*, 403–419.

[14] Y. Okamoto, E. Yashima, *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 1021–1043.

[15] E. Yashima, C. Yamamoto, Y. Okamoto, *Synlett* **1998**, 344–360.

[16] E. Yashima, *J. Chromatogr. A* **2001**, *906*, 105–125.

[17] Z. Chilmonczyk, H. Ksycinska, M. Mazgajka, H. Aboul-Enein, *Chirality* **1999**, *11*, 790–794.

[18] F. La Torre, R. Cirilli, R. Costi, R. Di Santo, R. Ferretti, B. Gallinella, A. Salerno, L. Zanitti, *Chromatographia* **2004**, *60*, 171–178.

[19] R. Cirilli, R. Ferretti, B. Gallinella, L. Turchetto, A. Bolasco, D. Secci, P. Chimenti, M. Pierini, V. Fares, O. Befani, F. La Torre, *Chirality* **2004**, *16*, 625–636.

[20] R. Silvestri, M. Artico, G. La Regina, A. Di Pasquali, G. De Martino, F.D. D'Auria, L. Nencioni, A.T. Palamara, *J. Med. Chem.* **2004**, *47*, 3924–3926.

[21] A.J. Edwards, P.J. Sweeney, D.G. Reid, J.M. Walker, N. Elshourbagy, C.E. Egwuagu, J.F. Young, C.L. Patton, *Chirality* **1996**, *8*, 545–550.

[22] H.Y. Aboul-Enein, I. Ali, *Fresenius J. Anal. Chem.* **2001**, *370*, 951–955.

[23] H.Y. Aboul-Enein, I. Ali, *J. Pharm. Biomed. Anal.* **2002**, *27*, 441–446.

[24] U. Vogt, P. Zugenmaier, *Ber. Bunsengs. Phys. Chem.* **1985**, *89*, 1217–1224.

[25] E. Yashima, C. Yamamoto, Y. Okamoto, *J. Am. Chem. Soc.* **1996**, *118*, 4036–4048.

[26] E. Yashima, M. Yamada, Y. Kaida, Y. Okamoto, *J. Chromatogr. A* **1995**, *694*, 347–354.

[27] T. Wang, R.M. Welson Jr, *J. Chromatogr. A* **2003**, *1015*, 99–110.

[28] L. Miller, C. Orihuela, R. Fronek, J. Murphy, *J. Chromatogr. A* **1999**, *865*, 211–226.

[29] E. Francotte, *J. Chromatogr. A* **2001**, *906*, 379–397.

[30] L. Miller, C. Orihuela, R. Fronek, D. Honda, O. Dapremont, *J. Chromatogr. A* **1999**, *849*, 309–317.

[31] C. Roussel, N. Vanthuyne, M. Serradeil-Albalat, J.C. Vallejos, *J. Chromatogr. A* **2003**, *995*, 79–85.

[32] R. Cirilli, R. Ferretti, B. Gallinella, L. Zanitti, F. La Torre, *J. Chromatogr. A* **2004**, *1061*, 27–34.

[33] M. Okamoto, *J. Pharm. Biomed. Anal.* **2002**, *27*, 401–407.

